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Cell surface expression of CCR5 and other host factors influence the inhibition of HIV-1 infection of human lymphocytes by CCR5 ligands

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Abstract

Several CCR5 ligands, including small molecules and monoclonal antibodies (MAbs), are being developed as therapies for infection with strains of human immunodeficiency virus type 1 (HIV-1) that use CCR5 for entry (R5 viruses). The efficacy of such therapies could be influenced by inter-individual differences in host factors, such as CCR5 expression levels. To study this, we used peripheral blood mononuclear cells (PBMCs) from humans and rhesus macaques. The half-maximal inhibitory concentrations (IC_{50}) of the small-molecule CCR5 ligands CMPD167, UK427,857 and SCH-D, and of the PRO 140 MAb, differ by >2 logs in a donor-dependent manner. We studied this variation by using flow cytometry to measure CCR5 expression on PBMCs from six of the human donors: the IC_{50} values of both SCH-D and PRO 140 correlated with CCR5 expression (R^2=0.64 and 0.99, respectively). We also determined the efficacy of the CCR5 ligands against HIV-1 infection of HeLa-derived cell lines that express CD4 at the same level but vary 2-fold in CCR5 expression (JC.48 and JC.53 cells). The moderately greater CCR5 expression on the JC.53 than the JC.48 cells was associated with proportionately higher median IC_{50} values for all four CCR5 ligands but not for a soluble CD4-based inhibitor or a non-nucleoside reverse transcriptase inhibitor. We conclude that differences in CCR5 expression on human PBMCs, which can be affected by *CCL3L1* gene dose, may influence the antiviral potency of CCR5 ligands *in vitro*, but other host factors are also likely to be involved. These host factors may affect the clinical activity of CCR5 inhibitors, including their use as topical microbicides to prevent HIV-1 transmission.

Keywords: CCR5 inhibitor; Viral entry; IC_{50}; Receptor density

Introduction

Several ligands of the CCR5 coreceptor are being evaluated for their potential use as therapy for infection with human immunodeficiency virus type 1 (HIV-1) (Westby and van der Ryst, 2005). They are also under consideration as topical microbicides to prevent HIV-1 sexual transmission (Veazey et al., 2005; Klasse et al., 2006). The rationale for such approaches is that CCR5 serves as the principal entry coreceptor for the most commonly transmitted strains of HIV-1 that predominate in the early years of infection (Moore et al., 2004). The binding of small molecules, monoclonal antibodies (MAbs) or chemokines (natural or modified) to CCR5 can interfere in several different ways with how the gp120 subunit of the HIV-1 Env complex interacts with the same receptor: the small molecules stabilize a CCR5 configuration that is recognized inefficiently by gp120, thereby acting as allosteric inhibitors (Billick et al., 2004; Dragic et al., 2000; Kazmierski et al., 2003; Kenakin, 2004; Seibert et al., 2006; Tsamis et al., 2003; Watson et al., 2005); MAbs are probably competitive inhibitors that impede the access of gp120 to CCR5 by a steric mechanism (Lee et al., 1999; Olson et al., 1999; Siciliano et al., 1999); and chemokines both down-regulate CCR5 and block gp120 binding competitively or non-competitively (Hartley et al., 2004; Signoret et al., 1997).

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The efficacy of several small molecule CCR5 inhibitors has now been demonstrated in human clinical trials (Fatkenheuer et al., 2005; Shen et al., 2004; Westby and van der Ryst, 2005) and in studies in SIV-infected monkeys (Veazey et al., 2003; Wolinsky et al., 2004). Significant patient-to-patient (or monkey-to-monkey) variations in the extent of viral load reduction have been observed during treatment with the small molecule inhibitors, but a mean viral load reduction of ~1.5 logs occurs over a 7–10 day dosing period (Fatkenheuer et al., 2005; Lalezari et al., 2005; McNicholl and McNicholl, 2006; Rosario et al., 2005; Veazey et al., 2003; Westby and van der Ryst, 2005). In the case of the SIV-infected monkeys, given the similarity of the infecting strain present in each animal, the variation in response is more likely to be dominated by host rather than viral factors. Because diverse HIV-1 strains are present within a patient population, both host and viral factors probably apply within infected people.

Here, we sought insights into the possible host factors that might influence the efficacy of CCR5 ligands *in vitro*. There are significant inter-individual differences in CCR5 expression, so one obvious parameter that could influence the efficiency of a CCR5 ligand is the CCR5 density on target cells. We used flow cytometry and the 2D7 MAb to measure CCR5 expression on peripheral blood mononuclear cells (PBMCs) from different human and macaque donors. At the same time, we determined whether CCR5 expression levels serve as a variable that influences the efficacy of small molecule inhibitors (CMPD167, UK427,857 and SCH-D) and the MAb PRO 140 against HIV-1 or SHIV infection via CCR5. The gp120-binding inhibitor PRO 542 (CD4-IgG2) was also studied for comparison with the CCR5 ligands, as was, in some experiments, the non- nucleoside reverse transcriptase inhibitor Efavirenz that acts against HIV-1 by an entirely different mechanism. Our results indicate that although variation in cell-surface CCR5 expression clearly influences how CCR5 ligands inhibit HIV-1 and SHIV infection of PBMCs from different human donors *in vitro*, other as yet undefined host factors are also operative. One factor that may affect cell-surface CCR5 expression is the dose of the gene encoding CCL3L1 (MIP-1α*P*), an HIV-1-inhibitory natural chemokine ligand of CCR5 that affects the extent to which CCR5 is down-modulated.

Results

The efficiency of CCR5 inhibitors against HIV-1 and SHIV infection of human and macaque PBMC is donor-dependent

We infected PBMCs from 32 human donors with the primary R5 HIV-1 isolates JR-FL and CC1/85 in the presence of the small molecule CCR5 ligands CMPD167, UK427,857 and SCH-D, and the PRO 140 anti-CCR5 MAb, to establish IC_{50} values for inhibition of infection (Figs. 1A, B). Viral growth was too low in the PBMC of two donors, one of which was homozygous for *CCR5 Δ32*, to allow IC_{50} determinations. For the remaining 30 donors, we used subsets according to whether sufficient numbers of cells were available. As an internal control and for comparative purposes, we concurrently determined the

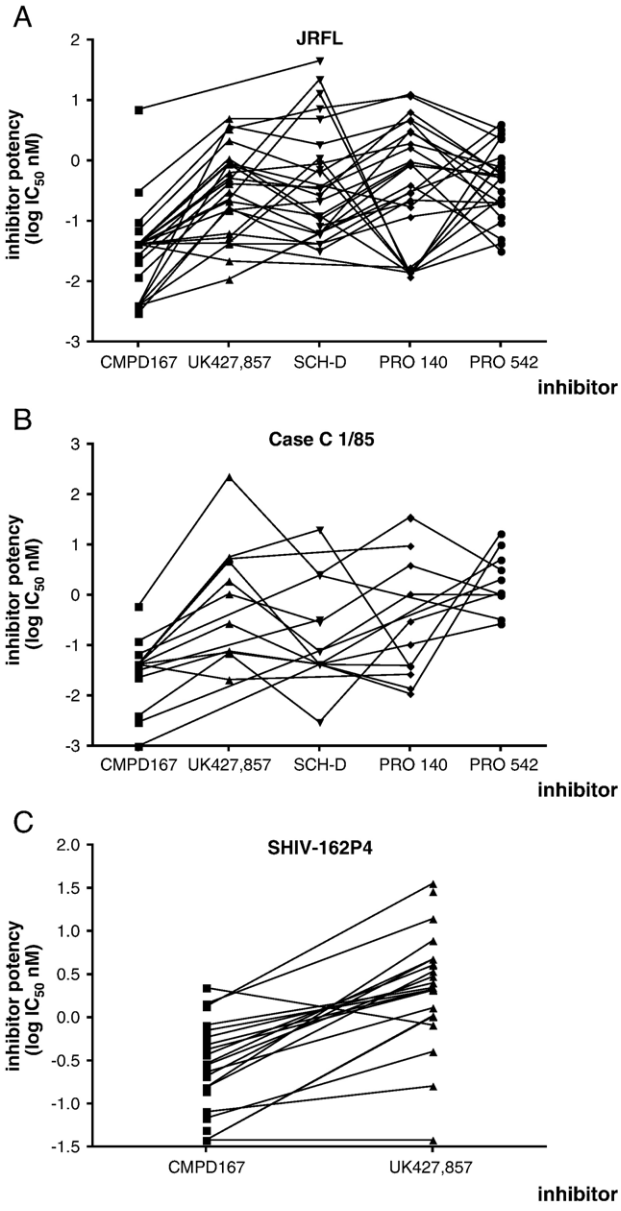

Fig. 1. The efficiency of CCR5 inhibitors against HIV-1 and SHIV infection of human and macaque PBMC is donor-dependent. Human PBMCs from different donors were infected with (A) HIV-1 JR-FL or (B) HIV-1 CC 1/85. The IC_{50} values for the indicated inhibitors were determined and their log values are plotted on the *y* axis. Data points obtained with different donor PBMCs are shown above each inhibitor. In (C), the experiment was of similar design, but involved infection of Rhesus macaque PBMC by SHIV-162P4. The percentage inhibition was calculated based on comparison with the average p24 concentration in 12 cultures without inhibitor. Each data point is an average of 2 replicate IC_{50} determinations derived from different but identical titrations. Inhibition of HIV-1 JR-FL was tested on PBMC from 27 to 30 donors. Among these, 20 to 28 gave sigmoid fits with $R^2 > 0.7$ for the different inhibitors. Only the IC values based on those fits are shown. Inhibition of HIV-1 CC 1/85 was tested on PBMC from 14 to 16 donors, and the 10 to 15 IC values based on sigmoid fits with $R^2 > 0.7$ are shown. PBMC from 23 to 25 macaques out of 29 gave fits with $R^2 > 0.7$. Because of some overlapping data points, not all individual IC_{50} values are visible. Thin lines in A, B and C connect data points for each donor.

IC_{50} values for the CD4-based, gp120-binding inhibitor PRO 542 using the same test viruses and the same donors. The replication efficiencies (p24 production) of the test viruses

ranged between 0.26 and 32 ng/ml in cells from the different donors.

However, the replication efficiency did not correlate strongly with the IC values ($R^2 < 0.4$). Furthermore, the IC_{50} values in themselves varied over a >100-fold range between donors for the CCR5 inhibitors, and for each of the test viruses. The potency of PRO 542 against both HIV-1 isolates varied by ~1 log less than this. Despite these wide variations, as a general rule we found that the relative potency of all the CCR5 inhibitors to be similar in cells from the same donor. However, there were exceptions: We observed that, sometimes, PBMCs from the same donor were more (or less) sensitive to a particular CCR5 inhibitor than might have been expected from the pattern observed with the other inhibitors. This was true of all the inhibitors: No inhibitor was obviously more or less subject than the others to the observed donor-dependent variation. A similar degree of variation was observed when CMPD167 and UK427,857 were used to inhibit SHIV-162P4 infection of PBMCs derived from 29 macaques (Fig. 1C). This *in vitro* variation may be related to the variable effect of CMPD167 on viral load among macaques infected with the SHIV-162P4 virus (Veazey et al., 2003).

Influence of CCR5 expression on sensitivities to CCR5 inhibitors in PBMC from different donors

For the next series of experiments, we studied PBMCs from six randomly chosen human donors in greater detail. We determined the IC_{50} values for inhibition of infection by the primary R5 HIV-1 isolates SB106, SB119 and AK103 for SCH-D (as a representative of the small molecule CCR5 inhibitors) and the PRO 140 MAb (Table 1). Because of the considerable variation that is inherent to the PBMC assay, we introduced the following features into our experimental design. First, the experiment was repeated three times; this allowed the determination of mean values (p24 production and IC_{50}) for each virus (and each donor). Second, for each viral strain, we normalized the p24 and IC_{50} values as explained in Materials and methods. This normalization enabled us to discern effects on susceptibility and inhibition that were truly specific to the PBMC donor.

The replication efficiencies of the three test viruses differed by ~4-fold between the six donors, as did the IC_{50} values for the two inhibitors (Table 1). CCR5 expression on the 6 PBMC samples varied over a 5-fold range (Figs. 2A–D). The relationships between CCR5 expression, the efficiency of HIV-1 infection and the IC_{50} values for inhibition by SCH-D and PRO 140 were then investigated (Figs. 2B–D). The correlation between the mean relative p24 production and CCR5 expression was weak ($R^2 = 0.56$), which implies that factors other than CCR5 levels might affect susceptibility to infection and production of progeny virus. The correlation between the IC_{50} for PRO 140 and CCR5 expression was strong ($R^2 = 0.99$), in part because of a single high data point. The correlations were similarly strong for the three individual viral strains used, with R^2 values of 0.99, 0.98 and 0.99. There was also a moderate correlation between the IC_{50} for SCH-D and CCR5 expression ($R^2 = 0.64$), but in this case the correlation varied among the three viral strains ($R^2 = 0.44$, 0.21 and 0.76). The pooling of data among strains appears to help in separating influences that are purely CCR5-dependent from those that are contingent upon the properties of the HIV-1 test isolate used.

None of the aforementioned variables was found to correlate with median fluorescence for CD4 (data not shown). However, in view of the possibility that CD4 and CCR5 levels might together influence the efficiency of HIV-1 entry and its inhibition (Platt et al., 1998), we also compared the production of p24 with the product of the CD4 and CCR5 median fluorescence values. For both p24 and the IC_{50} values for PRO 140, these correlations were weaker than those detected for CCR5 alone ($R^2 = 0.23$ and 0.95, respectively), whereas the correlation was stronger for the IC_{50} for SCH-D ($R^2 = 0.77$). The replication efficiencies did not correlate with the IC_{50} values ($R^2 < 0.24$), but the IC_{50} values for the two inhibitors were weakly correlated with each other ($R^2 = 0.49$).

We next explored the genetic basis for inter-individual differences in CCR5 cell surface expression among the six

Table 1

	Blood donor					
	35	53	55	61	67	68
Relative p24 [a]	1.4±0.017	1.1±0.022	1.3±0.064	0.36±0.036	1.0±0.053	0.83±0.021
Relative PRO 140 IC_{50} [a]	0.81±0.067	0.41±0.12	3.3±0.15	0.16±0.050	0.36±0.046	1.0±0.075
Relative SCH-D IC_{50} [a]	0.78±0.023	1.1±0.59	1.6±0.21	0.54±0.071	0.40±0.014	1.6±0.38
CD4 [b]	1200	860	3200	3100	1100	4500
CCR5 [b]	54	32	120	24	28	43
CCR5 genotype [c]	A/F2	F2/F2	A/F2	A/F2	C/G2	C/E
CCL3L1 copy number [d]	2	6	1	0	4	4

[a] The normalization and pooling of data for the three viral strains SB106, SB119 and AK103 were performed as follows. The mean of the three independent replicate assays for each viral strain was calculated. This was normalized to the mean value on the six donors for each strain. The mean value (±S.E.M.) for these relative p24 and IC_{50} values among the three strains are shown for each blood donor.

[b] The median cell-surface expression levels on intact cells, as determined by flow cytometry, are given for $CCR5^+$ cells in the case of CD4, and for $CD4^+$ cells in the case of CCR5. The values represent receptor expression levels on the day of infection. Additional FACS analyses of samples from the same cultures on three consecutive days yielded similar results.

[c] *CCR5* haplotypes were assigned as described previously (Gonzalez et al., 1999).

[d] *CCL3L1* copy number is the rounded number from the average of three independent runs.

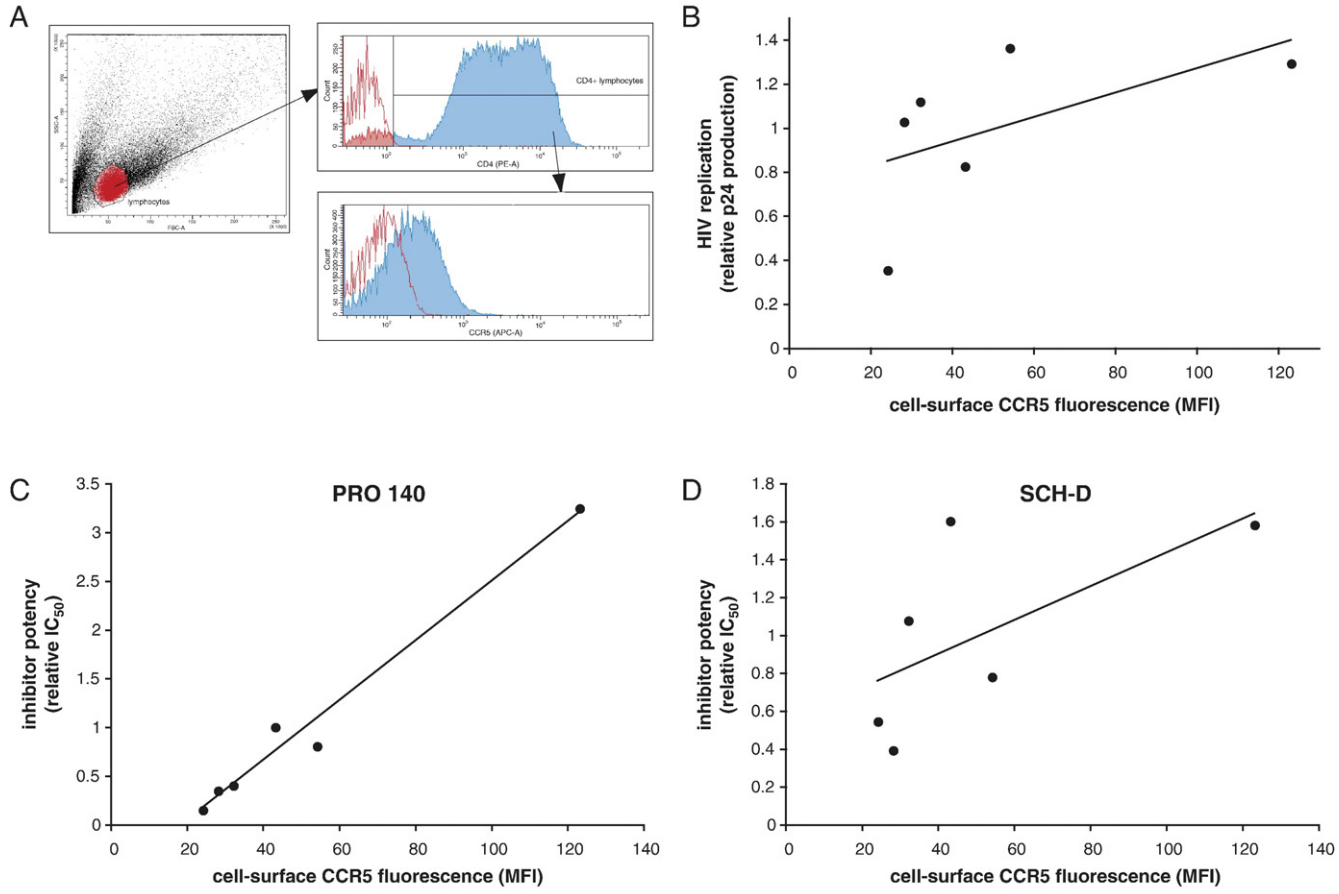

Fig. 2. The influence of CCR5 expression on the sensitivity to CCR5 inhibitors in PBMC from different donors. PBMCs from six human donors were infected with the primary HIV-1 isolates SB106, SB119 and AK103. The relative IC_{50} values for inhibition by PRO 140 and SCH-D were determined (see text). (A) The gating employed in the flow cytometry analyses is depicted. The population of lymphocytes was gated in the side-versus forward-scatter plot. The $CD4^+$ subpopulation of lymphocytes (blue in histograms) was then gated. (The red tracing in the histograms represents the isotype control.) The median CCR5 fluorescence intensities of these $CD4^+$ lymphocytes were used for the correlation. (B) The correlation between mean relative p24 production and CCR5 expression was weak, $R^2=0.56$. (C) The correlation of PRO 140 IC_{50} with CCR5 expression was strong, $R^2=0.99$. (D) The correlation of SCH-D IC_{50} with CCR5 expression was moderate, $R^2=0.64$.

donors that we had studied in greatest detail. The CCR5 haplotype pairs (genotypes) (Gonzalez et al., 2005) for the six donors are listed in Table 1. The single donor (#67) who was heterozygous for the CCR5 Δ32-carrying HHG*2 haplotype expressed CCR5 at a low level. Otherwise, there was no obvious association between these genotypes and CCR5 expression in this study (in which the sample size was very small). In contrast, the copy number of *CCL3L1* (the gene encoding MIP-1α*P*) (Table 1) correlated negatively but weakly with CCR5 expression; this was in accord with previous findings (Gonzalez et al., 2005). The R^2 values for the correlations between the *CCL3L1* gene dose and the mean and median values for cell surface expression of CCR5 on $CD4^+$ cells were −0.55 and −0.37, respectively. The percentage of $CCR5^+$ cells among the $CD4^+$ cells also showed a weak negative correlation with *CCL3L1* copy number; the R^2 value was −0.45. There was no association between the IC values for the two CCR5 ligands and *CCR5* genotype. This was an expected finding in view of the lack of any correlation between *CCR5* genotype and CCR5 expression in the few subjects studied.

Inhibition of infection of HeLa cell lines expressing different CCR5 levels

We also studied infection of the HeLa cell derivatives JC.48 and JC.53 by HIV-1 JR-FL Env-pseudotyped viruses, and its sensitivity to inhibition by CMPD167, UK427,857, SCH-D and PRO 140, and as controls, by PRO 542 and Efavirenz (Fig. 3). The median fluorescence intensity values for CCR5 expression on these cells were 4500 for JC.48 and 8700 for JC.53, which is approximately proportional to 5.0×10^4 and 1.3×10^5 molecules per cell, respectively, as determined by Scatchard analysis of radio-labeled ligand binding to earlier passages of the same cells (Platt et al., 1998). These levels of CCR5 expression per cell are ~100-fold greater than the range typically observed on human PBMCs. Despite this difference, the IC_{50} values for the JC.48 cells were not higher than those for the PBMC. However, the cell surface density of CCR5 rather than the total number of CCR5 molecules per cell might have the greater influence on the potency of an inhibitor. HeLa cells are larger than human $CD4^+$ T-cells (2-fold greater median forward scatter signal by flow cytometry). Hence the densities of CCR5 on the two cell

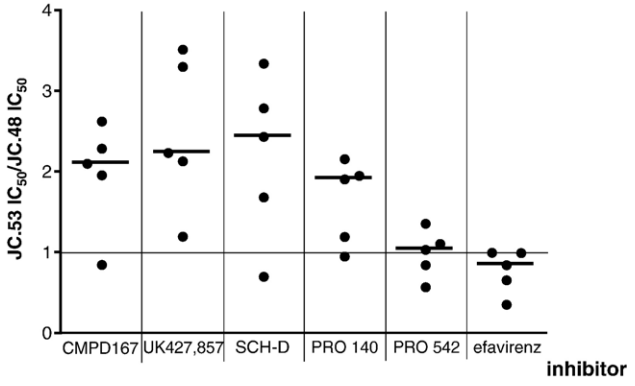

Fig. 3. The sensitivity of HIV-1 infection to CCR5 inhibitors in HeLa cell lines expressing different amounts of CCR5. HeLa cell lines expressing high (JC.53) or low (JC.48) levels of CCR5 were infected with HIV-1 JR-FL pseudotype virus. The cells or virus were preincubated with CMPD167, U427,857, SCH-D, PRO 140, PRO 542 or Efavirenz at a range of concentrations. After 72 h, the cells were lysed, the luciferase activity was measured and the IC_{50} values for inhibition on the two cell lines were determined. The symbols each represent the ratio of IC_{50} values on the JC.53 cells over those on the JC.48 cells, calculated from five independent experiments. Horizontal bars indicate medians.

types are likely to be more similar than the total number of molecules.

The JC.53/JC.48 IC_{50} ratios were calculated for all inhibitors in five independent experiments. The ratios for CMPD167, UK427,857, SCH-D, PRO 140 and PRO 542 were then each compared with that for Efavirenz by the one-tailed Mann–Whitney *U* test. For Evarirenz and PRO 542, these ratios were ~1.0, implying that the activity of these inhibitors is CCR5-independent, as expected. The median JC.53/JC.48 IC_{50} ratios for the CCR5 ligands were significantly greater than for Efavirenz ($p=0.016$; $p=0.0040$; $p=0.028$ and $p=0.016$, respectively) but not for PRO 542 ($p=0.15$). The JC.53/JC.48 IC_{50} ratios for all the CCR5 ligands, taken together, were also significantly higher than the ratios for the two CCR5-independent inhibitors, again taken together ($p=0.0002$, one-tailed, Mann–Whitney *U* test).

Discussion

Our goals in this study were 2-fold. First, to assess the degree of variation in antiviral potency of CCR5 inhibitors when they were tested using PBMCs from multiple human donors; second, to determine whether CCR5 expression levels affected the potency of the CCR5 inhibitors. Intuitively, there might be expected to be a linear relationship between CCR5 availability on the cell surface and the concentration of an inhibitory ligand required to prevent the receptor from being used by a fixed amount of HIV-1. More specifically, a greater density of unoccupied CCR5 in the vicinity of an attached virion would favor the recruitment of CCR5 into a fusion complex. There is abundant evidence for donor-dependent variation of CCR5 expression, even among donors who do not possess the CCR5-Δ32 allele. Early studies identified an ~20-fold variation in CCR5 expression on PBMC from human donors who lack this mutation (Moore, 1997; Wu et al., 1997). Much of this variation is probably attributable to polymorphisms in the non-coding regions (e.g. promoter) of *CCR5*, which are known to have a substantial influence on the rate of disease progression in HIV-1 infected people (Martin et al., 1998; Mummidi et al., 1998).

There are two clear limitations of this study: the first is the well-known imprecision of the PBMC assay for HIV-1 replication, the second is the limited number of replicate assays that can be performed using cells from a blood donor sample. Intra-assay variation of this type when using cells from a single donor will necessarily blur inter-assay comparisons made using cells from multiple donors. As outlined above, we took several steps to minimize intra- and inter-assay variation and the influence of distinct viral strains; specifically, we conducted multiple repeat experiments, we pooled data derived from three different viral strains, and we normalized the data for comparative purposes. Nonetheless, identifying subtle factors that could affect the sensitivity of CCR5 inhibitors remains problematic in the PBMC assay. Within the context of these limitations, we observed that that the apparent influence of CCR5 surface expression levels on the inhibitory efficiency of CCR5 ligands is greater than the influence CCR5 levels have on the susceptibility of PBMC to infection by R5 viruses.

By comparing human PBMCs with a pair of HeLa-cell-derived cell-lines that express different amounts of CCR5, part of the donor-dependent variation in the efficiency of entry inhibition via CCR5 in PBMC assays could be shown to be attributable to CCR5 expression. On the lymphocyte cell surface, CCR5 expression varied over a 5-fold range. The IC_{50} values, which correlated with the CCR5 expression level, varied by 20-fold for PRO 140 and by 4-fold for SCH-D. Furthermore, on the HeLa cells, a 2-fold difference in CCR5 expression (albeit at a much higher absolute level compared to lymphocytes) translated into proportionate differences in median IC_{50} values specifically for the CCR5 ligands but not the other inhibitors. Overall, we conclude that unidentified factors other than CCR5 expression levels (as measured using MAbs like 2D7) are likely to modulate the inhibitory efficiency of CCR5 ligands. The activated PBMCs also vary in CD4 expression (5-fold in median m.f.i. on $CCR5^+$ cells, Table 1), while the CD4 levels are the same on the two HeLa cell clones. CD4 expression does not, however, seem to be a strong modulating influence on inhibitor sensitivity; correlations with IC_{50} values did not substantially change when the CCR5 levels were multiplied by the CD4 levels (we tested these correlations because, in receptor-transfected HeLa cells, variations in the surface levels of CCR5 and CD4 have been shown to compensate for each other's effects on susceptibility (Kuhmann et al., 2000; Platt et al., 1998)). Nevertheless, we note that receptor level effects can be interconnected: the soluble CD4-based, coreceptor-independent inhibitor PRO 542 was somewhat less efficient at blocking infection of the HeLa cells with the higher CCR5 level than of the cells expressing less CCR5.

As noted above, the CCR5 ligand that probably acts competitively with Env, i.e. the PRO 140 MAb, had a modestly stronger dependence on the CCR5 expression level on the $CD4^+$ T cells than a small molecule inhibitor with a non-competitive mechanism of action. However, no such difference was ob-

served with the HeLa cells. This suggests that our understanding of how small molecule inhibitors might function is likely to be incomplete, as is our knowledge of how their action is influenced by donor-specific factors other than simply the expression of CCR5 protein on the cell surface. For example, in our flow cytometry assays for CCR5 expression, we used the generally accepted standard reagent for staining CCR5, the 2D7 MAb. This MAb probably does not react uniformly with all possible isoforms and conformers of CCR5 that could be present on the cell surface and relevant to HIV-1 entry. It has been well documented that different MAbs to different CCR5 epitopes generate different estimates of CCR5 expression on the surface of PBMC (Lee et al., 1999). Conceivably, how HIV-1, small molecule inhibitors and 2D7 recognize these different forms of CCR5 may differ, potentially confounding any possible correlations. We note, however, that variation in potency due to unidentified cellular factors applies to both PRO 140 and the small molecules alike. There is a report that the sensitivity of different HIV-1 strains to small molecule CCR5 ligands varies more than to PRO 140 (Safarian et al., 2006). This may be because small molecules and MAbs interfere differently with the entry process: small molecules have been suggested to block V3-dependent binding to the second extracellular loop of CCR5, whereas MAbs impede the recruitment of co-receptors into fusion complexes and conformational changes within these complexes (Safarian et al., 2006). How variation in CCR5 levels affects these different stages of entry, and their inhibition, requires further study.

Studies in cell lines transfected to stably express different CCR5 levels have been used to demonstrate that CCR5 expression affects the efficiency of CCR5 ligands. For example, CCR5 expression was varied on $CD4^+$ T-Rex/CCR5 cells using a tetracycline-regulated promoter, and measured using 2D7. HIV-1 infection efficiency was independent of CCR5 expression, but lower concentrations of the small molecule CCR5 inhibitor TAK-779 were required to inhibit infection of the cells expressing the lowest CCR5 level, compared to the other two lines (Reeves et al., 2002). Similarly, TAK-779 was found to be ~15-fold more potent on HeLa-CD4/CCR5 cells bearing a limiting CCR5 concentration (700–2000 receptors per cell) than on cells with an excess of CCR5 (10,000–20,000 per cell) (Platt et al., 2005). A third study compared U87/CD4/CCR5 and NP2/CD4/CCR5 cells, which are both derived from the astroglioma lineage (Willey et al., 2005). The CCR5 expression levels were reported to be "far greater" on the NP2 cells, although no values were given. The inhibitory concentrations of several different CCR5 ligands were ~10-fold higher with the NP2 cells (high CCR5) compared to the U87 cells (low CCR5), irrespective of the test virus. Similar results were obtained using HeLa cells expressing high or low levels of CD4 or CCR5, the potency of the CCR5 ligands again being inversely proportional to the CCR5 expression level, but independent of CD4 expression. For the small molecule inhibitor UK400,343, the shift in the inhibition curves between the high- and low-CCR5 expressing cells was ~100-fold when CD4 expression was high (Willey et al., 2005).

Our results using human PBMCs are consistent with all the above observations, but we observed rather smaller effects. This is partially because the extent of CCR5 expression on PBMC from different donors varies over a smaller range than among some high-CCR5 and low-CCR5 cell lines. A second factor is the aforementioned assay imprecision that blurs correlations when PBMC are used, which is a lesser problem with cell line-based assays.

An additional factor to consider is how much CCR5 expression affects the efficiency of HIV-1 entry, because CCR5 ligands serve to reduce the effective CCR5 concentration available on the cell for use by HIV-1. In one early study using HOS-CD4 cells expressing high or low CCR5 levels, a moderate (7-fold) difference in CCR5 expression (measured using 2D7) resulted in a much greater difference (30- to 80-fold) in the amount of virus produced after a single round of replication. This difference was attributed to post-entry effects, and not to alterations in the efficiency of HIV-1 entry (Lin et al., 2002). However, entry was measured using cytoplasmic p24 uptake, which can be problematic for discriminating between productive and non-productive infection (Schwartz et al., 1998). When CCR5 expression on peripheral blood $CD4^+$ T-cells from HIV-1-infected and control individuals was measured using quantitative flow cytometry and MAb 2D7, it was found to vary over a 4–5-fold range (Reynes et al., 2001). On average, cells from slow progressors expressed less CCR5 than normal progressors (7400 compared to 11,000 molecules per cell), while uninfected individuals expressed CCR5 at levels similar to normal progressors (Reynes et al., 2001). The relationship between *in vivo* viral load and *ex vivo* measurements of CCR5 expression was log-linear in nature, implying that minor variations in CCR5 expression could have a much greater effect on virus production. A similar inference can be drawn from the use of siRNA to silence CCR5 expression *in vitro*: again, a linear decrease in CCR5 expression resulted in a logarithmic decline in HIV-1 infection efficiency (Butticaz et al., 2003). This observation has implications for the activity of more conventional inhibitors that bind to CCR5 directly, and by doing so, reduce the effective concentration of the co-receptor that is available for usage by HIV-1. An explanation might lie in the multiplicity of CCR5 proteins required for formation of a functional fusion complex (Kuhmann et al., 2000).

We detected an inverse association between the copy number of *CCL3L1*, but not the CCR5 genotype, and CCR5 expression levels. This inverse association is consistent with previous findings (Gonzalez et al., 2005). The *CCL3L1* copy number is a whole-number variable (e.g. zero, one, two copies), whereas there are a large number of *CCR5* genotypes (Gonzalez et al., 2001). In this light, when the sample size is small, it is easier to detect an association for *CCL3L1* copy number than for *CCR5* genotype. Our findings also reinforce the notion that *CCL3L1* gene dose might be a key determinant of CCR5 expression levels, which could therefore not only affect an individual's susceptibility to HIV-1/AIDS but also inter-individual differences in responses to new, CCR5-targeted therapies.

Virus-dependent influences on the potency of CCR5 inhibitors *in vitro* have also been identified. R5 isolates obtained from early in the course of HIV-1 clinical infection have been found in some studies, but not in others, to be more

sensitive to various CCR5 ligands when tested in various cell types, including PBMCs (Gray et al., 2005; Jansson et al., 1999; Koning et al., 2003; Olivieri et al., 2007; Rusert et al., 2005). Generally, viruses from early in infection required higher levels of CCR5 and/or CD4 than those isolated in later years, which have a reduced dependence on both CD4 and CCR5 levels (Gray et al., 2005). The dependence of HIV-1 infection on the extent of CD4 or CCR5 expression on target cells is well established (Kuhmann et al., 2000; Platt et al., 1998). Overall, these sets of observations are consistent with the acquisition of an increased affinity for CCR5 during HIV-1 evolution in an infected individual, perhaps driven by the preferential loss of cells expressing higher CCR5 levels. However, the extent to which virus-specific factors will affect the clinical performance of CCR5 inhibitors is hard to predict, both in absolute terms and relative to host-dependent variables. The development of resistance through viral evolution under drug-selection pressure will, of course, compromise inhibitor efficacy, but this is a different topic from the baseline sensitivity issues we are considering here. Host-dependent variation in CCR5 expression levels on relevant target cells could also influence the efficiency with which topically applied CCR5 inhibitors impair the sexual transmission of HIV-1, if and when they are used as vaginal or rectal microbicides (Veazey et al. 2005; Klasse et al., 2006).

Materials and methods

Inhibitors and MAbs

The small molecule CCR5 inhibitors CMPD167, UK427,857 and SCH-D have all been described elsewhere (Marozsan et al., 2005; Tagat et al., 2004; Veazey et al., 2003; Westby and van der Ryst, 2005). SCH-D and UK427,857 are now known as vicriviroc and maraviroc, respectively. The PRO 140 MAb binds to CCR5, whereas PRO 542 is a tetravalent, IgG-fusion protein that contains the first and second extracellular domains of CD4 and inhibits HIV-1 infection by binding to gp120 (Allaway et al., 1995; Olson et al., 1999). The NNRTI Efavirenz was a gift from Bill Olson (Progenics).

Virus isolates

The R5 primary isolate JR-FL was obtained from the NIH AIDS Reagent Repository (http://www.aidsreagent.org/); CC 1/85 from Ruth Connor (Connor et al., 1997; Trkola et al., 2002); SB106, SB119, AK103 from Alexandra Trkola (Rusert et al., 2005). The simian-human hybrid virus SHIV-162P4 (Harouse et al., 2001; Tan et al., 1999; Veazey et al., 2003) was obtained from Ron Veazey and subsequently expanded in macaque PBMC.

PBMC infectivity assay

Human lymphocytes were prepared from leukopacks obtained from the New York Blood Center (New York, NY). The resulting PBMC were depleted of $CD8^+$ cells by use of the RosetteSep $CD8^+$ cocktail (StemCell Technologies, Vancouver, BC, Canada) during Ficoll density gradient separation, as specified by the manufacturer. The PBMC were cultured in lymphocyte medium (LM: RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Cellgro, Herndon, VA), 100 μg/ml Penicillin/Streptomycin, 2 mM L-glutamine and 50 U/ml IL-2 (NIH AIDS Research and Reference Reagent Program, contributed by Hoffman-LaRoche, Inc.). Equal parts of each PBMC culture were stimulated with either 5 μg/ml of phytohemagglutinin (PHA; Sigma, St. Louis, MO) or with supernatant from the OKT3 hybridoma (anti-CD3 stimulation). After 3 days, the cells from the two different stimulation cultures were combined into one culture for each donor. The PHA and OKT3 were removed by washing and replaced with lymphocyte medium for the remainder of the time in culture (Ketas et al., 2003). PBMC from rhesus macaques were isolated by the same procedure as used for human PBMC, except that the Ficoll was diluted by 10% with PBS and there was no depletion of $CD8^+$ cells.

PBMC infectivity assay

After 3 days of stimulation, the washed PBMC were seeded at 100 μl per well into a 96-well plate (Becton Dickenson) at 1.4×10^6 cells/ml in LM, then incubated with a CCR5 inhibitor diluted 5-fold in 10 serial steps, or with media, for 1 h before addition of virus. For studies with PRO 542, the inhibitor was mixed with the virus in a separate plate for 1 h before addition of the mixture to the cells. HIV-1 replication was measured by p24 antigen ELISA on day 7, as previously described (Ketas et al., 2003). Residual p24 from the input virus was measured and subtracted. Net p24 production in the test wells was compared to that in the control wells (no inhibitor, defined as 100%). Similarly, SHIV replication was measured by p27 antigen ELISA kit (Zeptometrix, Buffalo, NY), according to the manufacturer's instructions. The IC_{50} values were determined on days 6–14, depending on viral growth in control cultures.

For one series of experiments on PBMC from six donors, the cells were isolated and stored in aliquots at −80 °C in RPMI 1640 medium containing 50% FBS and 10% dimethyl sulfoxide (Sigma, St. Louis, MO), to allow subsequent infectivity assays to be repeated using the same cells. The cells were thawed in an excess of RPMI 1640 containing 50% FBS, then stimulated and infected as described above.

Flow cytometric analysis of CD4 and CCR5 on $CD8^+$-depleted PBMC

CD4 was detected with a PE-conjugated anti-CD4 MAb (clone SK3, BD Immunocytometry Systems, San Jose, CA), CCR5 with an APC-conjugated anti-CCR5 MAb (clone 2D7, BD Pharmingen, San Jose, CA). PE- and APC-conjugated isotype controls MAbs were obtained from BD Pharmingen. For staining with MAbs, 1×10^6 $CD8^+$-depleted PBMC were suspended in 50 μl of cytometry buffer (RPMI 1640 without phenol red + 10% FBS + 0.1% NaN_3). An aliquot (10 μl) of each of the anti-CD4 and anti-CCR5 MAbs or the isotype control MAbs was added for 1 h at 4 °C. The cells were washed three

times with 200 μl of cytometry buffer, with a 5 min incubation at 4 °C prior to each wash. After the final wash, the cells were resuspended in 200 μl of fixing solution (2% w/v paraformaldehyde, 2% w/v sucrose in phosphate buffered saline) and incubated for 15 min at room temperature before the addition of 200 μl of cytometry buffer. Fixed cells were stored at 4 °C until they were analyzed on an LSRII cytometer (BD Immunocytometry Systems). All of the samples used in this analysis were analyzed in the same cytometry run, allowing a direct comparison the MFI values. The lymphocyte population was identified on the basis of forward (FSC) and side scatter (SSC) parameters. The gate was drawn so as to exclude monocytes (the cells with greater FSC and SSC in Fig. 2A), and typically included 70% to 80% of total cells. The $CD4^+$ cell population was identified on the basis of specific staining for CD4 (see Fig. 2A). The broadening of the CD4 peak seen in Fig. 2A was a likely result of the early time after activation, as it resolved into a narrow peak at a high MFI by day 5 post activation, similar to what was seen on unactivated cells (results not shown). The median fluorescence intensities were calculated within the FACSDiva software (BD Immunocytometry Systems) for the $CD4^+$ lymphocyte population, after subtraction of the background MFI values on cells from the same donor that were stained with isotype control antibodies. Receptor-expression levels on PBMC samples were analyzed on the day of infection, i.e. on day 3 of the culture.

CCR5 and CCL3 genotyping

Genomic DNA was isolated from all the donors by using a QIAamp DNA mini kit (Qiagen, Valencia, CA). Polymorphisms in *CCR5/CCR2* were detected as described previously (Gonzalez et al., 1999), and included the polymorphisms at *CCR5*-29, 208, 303, 627, 630, 676, 927 in the *CCR5* promoter region, the *CCR5*-Δ32 in the *CCR5* coding region and the CCR2-64V/I from the *CCR2* coding region. All the sequences for the primers and probes and methods are available upon request from Dr. S. K. Ahuja. Haplotypes were assigned as described previously (Gonzalez et al., 1999). *CCL3L1* copy number was screened as reported elsewhere (Goldstein et al., 2000).

Infection of HeLa cell lines by Env-pseudotyped viruses

The HeLa cell-derived clones JC.48 and JC.53 expressing CD4 and different levels of CCR5 were a gift from David Kabat (Platt et al., 1998). HIV-1 JR-FL Env-pseudotyped viruses were harvested from 293T cells (NIH) co-transfected with the NL-Luc backbone plasmid (Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH; contributed by N. Landau) and the JR-FL *env* plasmid (Beddows et al., 2005). The cells or the inoculum viruses were incubated with titrated inhibitors, as in the PBMC infectivity assay. After 72 h of culture, the cells were lysed and the luciferase activity analyzed with the Bright-Glo Luciferase Detection Kit (Invitrogen), according to the manufacturer's instructions. The experiment was repeated five times, with all the results being depicted in Fig. 3.

Statistical and mathematical analysis

IC_{50} values were calculated from sigmoid curve-fits (Prism, GraphPad). All IC_{50} data presented fulfill the criterion of $R^2 > 0.7$; those in the detailed analysis of six donors had, in addition, 95%-confidence intervals <10-fold. Correlations were calculated and statistical comparisons (Mann–Whitney test) were carried out using Prism (GraphPad).

In the detailed analysis of PBMC infection by the three primary isolates, SB106, SB119 and AK103, we adopted a normalization procedure in order to reduce confounding variation attributable to differences among the stocks of the three viral strains. Each viral strain was tested in triplicate in order to control experimental variation. The mean p24 and IC_{50} values of these three replicates were calculated. Then the mean p24 and IC_{50} values among the six PBMC donors were calculated for each strain. These means were used to normalize the values for each viral strain. We then pooled these relative p24 and IC_{50} values for the three viral strains and calculated a mean for each donor. We thereby obtained relative donor-specific values for both susceptibility and inhibition that were minimally affected by the different contents of infectious virus in the stocks of the three strains.

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